Techniques for Inoculation of Peanut with *Sclerotium rolfsii* in the Greenhouse and Field[1]

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ABSTRACT

Four greenhouse experiments and a field trial were conducted in 1994 to determine the most effective technique for inoculation of peanut (*Arachis hypogaea* L.) with *Sclerotium rolfsii* Sacc. The cultivar Florunner was grown in the greenhouse in 20-cm-diameter pots and inoculated 48 d after planting (DAP). Plants in the field experiment were grown in plots 2.7 m long on 0.9 m centers, thinned to 11 plants per plot, and alternate plants were flagged for inoculation at 48 DAP. Inoculation techniques for all experiments were (a) a germinating sclerotium on a 1-cm-diameter agar disk [potato dextrose agar (PDA)] appressed to the base of each central stem; (b) mycelia of a composite of six isolates growing on sterilized oat seed placed on the soil near the base of each central stem (20 g/pot) in the greenhouse or in the center of rows (a full 150 mL beaker of inoculum per row) in the field; (c) 2-3 mL of a PDA slurry with actively growing mycelia applied to the base of each central stem; (d) mycelia on toothpicks impregnated with potato dextrose broth (PDB) inserted into the base of each central stem; (e) a toothpick with mycelia [as in (d)] inserted into the soil near the base of each central stem; and (f) mycelia on PDB-impregnated clothespins clamped around the base of each central stem. The most effective methods in all experiments were the agar disk technique (a) and the clothespin technique (f). The oat inoculum technique (b) was only slightly less effective than techniques a and f. Techniques a and f have the advantage of allowing distinct single-plant inoculation. The oat inoculum technique allows the use of a composite of multiple isolates and inoculation of entire rows of plants or large areas of a field. The other techniques (c, d, and e) produced significantly ($P \leq 0.05$) less disease. Inoculation with mycelia cultured on toothpicks inserted into stems (d) worked well sometimes but lacked consistency. This technique wounds plants and may bypass some possible natural defense mechanisms. We prefer the clothespin technique for evaluation of individual plant resistance because it is rapid and reliable. This is the first report for use of this technique for inoculation of peanut with *S. rolfsii*.

Key Words: *Arachis hypogaea*, groundnut, white mold, southern stem blight, sclerotium rot.

Sclerotium rolfsii Sacc. is an economically important pathogen in warm, moist climates worldwide, causing diseases on more than 500 species of plants (1). This fungus causes a stem and pod rot of peanut. Pod yields may be reduced as much as 2.9% per disease locus (3) and overall losses from stem rot are estimated at 7-10% annually in the southeastern U.S. (11). Virulent strains of the pathogen grow rapidly and produce large amounts of oxalic acid and cell wall-degrading enzymes (13). Decaying organic matter provides a food base for growth enhancing the ability of the fungus to infect living plants (10). Disease incidence is higher when soil and debris are brought into contact with peanut plants (4). The peanut canopy provides a warm, moist environment conducive to the growth of the pathogen and initial infection by *S. rolfsii* often occurs on the central stems (1, 7, 8). Sclerotia of the fungus may survive in soil for long periods (1) and are the primary inoculum of *S. rolfsii*. Remoistened peanut hay produces small amounts of methanol and other volatile compounds that stimulate sclerotial germination (2, 14). Sclerotia may eruptively germinate (many cells produce mycelia at the same time) in the presence of volatile compounds and infect without a food base (12).

Sclerotium rolfsii grows well on organic matter and produces sclerotia depending on the nutritional status of the substrate. The pathogen produces abundant mycelia on seed of oat (*Avena sativa* L.) which have a high test weight and many sclerotia on oat seed with low test weight (4). Inoculum of *S. rolfsii* is frequently grown on oat seed and used to infest peanut fields in research studies (6, 16, 17). Peanut breeders often evaluate breeding lines and cultivars under natural inoculum levels (5, 6) or with artificial infestations using laboratory-grown mycelial or sclerotial inoculum (6, 16, 17).

Cultivars of peanut with resistance or tolerance to southern stem rot are needed, but screening for resistance in the field is complicated by the nonuniform spatial distribution of sclerotial inoculum (15). Consistent, reliable data to confirm the resistance of cultivars or breeding lines is difficult to obtain in fields with natural infestations of *S. rolfsii*. This is evidenced by the fact that the stem rot resistance of the cultivar Southern Runner (9) was not reported until 5 yr after its release (6). Evaluation of genotypes for resistance to *S. rolfsii* would be more reliable if uniform methods of inoculation were available. The objective of this research was to compare several inoculation techniques in the greenhouse and in the field to determine the most effective methods for identifying *S. rolfsii*-resistant genotypes.

Materials and Methods

Greenhouse Experiments. Four greenhouse experiments (GH1-GH4) were planted at the North Florida Research and Education Center (NFREC) on 10 May, 26 May,

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8 June, and 10 Aug. 1994. Pots (20 cm diameter) were filled with a commercial potting media (Metromix 320, W. R. Grace and Co., Cambridge, MA) premixed with 5-10-15 fertilizer (5 g/pot), and the insecticide disulfoton (0.07 g/pot). Three seeds of cv. Florunner were treated with *Bradyrhizobium* inoculant and planted in each pot. Pots were thinned to one vigorous plant/pot 3 wk after planting. Plants were inoculated with *S. rolfsii* on either 48 or 49 DAP using isolates that had been pretested in the greenhouse for virulence against peanut. Plants were covered with plastic bags after inoculation and kept shaded for 48 hr to maintain high humidity and promote the establishment of disease. Greenhouse temperatures ranged from 21 to 35 C. The experimental design for each greenhouse experiment was a randomized complete block with 10 replicates of each treatment and appropriate noninoculated controls.

Field Experiment. The field experiment was planted in a Chipola sandy loam soil on 10 May at the NFREC, Marianna, FL. Plants were inoculated on 27 June (48 DAP) and assessments for disease were performed on 28 June, 5 July, 18 July, and 2 Aug. Seed of Florunner were planted in plots 2.7-m long with rows on 0.9-m centers. Six seeds were planted for each 30 cm of row and plants were thinned to 11 plants/plot after 3 wk of growth. Management practices for fertilization, weed control, and insect control were in accordance with recommended practices for the lower southeastern peanut belt. Chlorothalonil (1.26 kg ai/ha) was applied seven times to control leaf spot diseases. Gypsum (563 kg/ha) was applied to all plots during early pegging (40 DAP); otherwise no agrichemicals that are known to suppress stem rot were used. Alternate plants in each plot were marked with a surveyor's flag for inoculation and assessment. Ten replications of each inoculation technique were used in a randomized complete block design and five plants were inoculated in each plot. A noninoculated check plot was included in each replicate. The experiment was irrigated with 1.3 cm of water before inoculation and similarly on 2 d thereafter to provide conditions conducive for infection.

Inoculation Techniques. Inoculation techniques were as follows: (a) a germinating sclerotium on a 1-cm-diameter agar disk [potato dextrose agar (PDA)] appressed to the base of each central stem, (b) mycelia of a composite of six isolates growing on sterilized oat seed placed on the soil near the base of each central stem (20 g/pot) in the greenhouse or in the center of rows (a full 150-mL beaker of inoculum per row) in the field, (c) 2-3 mL of a PDA slurry with actively growing mycelia applied to the base of each central stem, (d) mycelia on toothpicks impregnated with potato dextrose broth (PDB) inserted into the base of each central stem, (e) a toothpick with mycelia [as in (d)] inserted into the soil near the base of each central stem, and (f) mycelia on PDB-impregnated clothespins clamped around the base of each central stem.

Inoculum for the agar disk technique was prepared using sclerotia produced on plates of PDA (Difco Laboratories, Detroit, MI). Sclerotia of isolate SR 18 (obtained from peanut at Marianna, FL during 1988) were dipped in 95% ethanol for 1 min, placed on PDA plates 2 cm apart, and allowed to germinate for 48 hr. A 1-cm-diameter agar disk with the sclerotium in the center was cut with a sterile cork borer and appressed to the base of the central stem of designated plants.

Oat inoculum was prepared by imbibing oat seed with water, freezing overnight, and then autoclaving for 120 min on each of two successive days. The seed then were distributed into sterile plastic bags under a transfer chamber and inoculated with 1-d-old cultures of *S. rolfsii* grown on PDA. Individual bags were inoculated with one of six isolates of *S. rolfsii* (SR2, 18, 19, 38, 55, or 65). SR2 was isolated from peanut at Yoakum, TX in 1987 by Dr. D. H. Smith; SR19 from soybean [*Glycine max* (L.) Merr.] at Quincy, FL in 1990; SR38 from peanut at Marianna, FL in 1991; SR55 from peanut at Quincy, FL in 1991; and SR65 from peanut at Marianna, FL in 1992. Mycelia were allowed to grow for 3 wk to thoroughly infest seed prior to inoculation of plants and equal parts of seed with each isolate were mixed to make a composite inoculum. Oat seed inoculum was mainly mycelial with very few sclerotia. In greenhouse tests, 20 g of oat inoculum were placed in each pot near the central stems. Some of the oat seed inoculum in experiment GH3 became contaminated with a *Trichoderma* sp. Therefore, an additional greenhouse experiment (GH4) was conducted. In the field test oat inoculum (a full 150 mL-beaker) was scattered down the center of each row and moved beneath the foliar canopy by dragging a weighted cloth bag through the foliage.

The mycelial slurry inoculum (PDA slurry) was made by blending 2% sterile PDA slurry with mycelium of 3-4-d-old cultures of SR18 grown on PDA (three plates of mycelium/L of slurry). Slurry (2-3 mL) was applied to the base of the central stems using a 50-mL self-refilling repetitive syringe (Fisher/Wheaton Sci. Prod., Pittsburgh, PA).

Toothpick and clothespin inocula were produced by boiling wooden toothpicks and clothespins (substrate) in water twice to remove tannins. These substrates were then impregnated with PDB (Difco Laboratories, Detroit, MI) by autoclaving together in beakers. The excess broth was decanted and the substrate was inoculated with SR18. The fungus was grown on the substrate for 1 wk before use. Toothpicks were inserted into central stems about 1 cm above the soil surface (technique d) or placed upright in soil *ca.* 1 cm deep and 1 cm from the central stems (technique e). Clothespins were clamped *ca.* 1 cm above the soil around the central stems using the rounded depressions in the clamp end so that plants were not wounded (technique f).

Noninfested toothpicks were inserted into stems as an untreated check for comparison against technique d since this technique is a form of wound inoculation. Appropriate checks (10 each) without inoculation also were used in each experiment.

Disease Assessments. In greenhouse experiments, the first assessment of stem rot severity was made either 4 or 5 d after inoculation and every 2 or 3 d thereafter for a total of six (GH1-GH3) or seven (GH4) assessments. Four assessments were made in the field test beginning 1 wk after inoculation and at 2-wk intervals thereafter. Untreated checks were available to monitor disease caused from natural inoculum. A 1-5 scale was used for all disease assessments—1 = a healthy plant, 2 = lesions on stems only, 3 = up to 25% of the plant symptomatic (wilted, dead, or dying), 4 = 26-50% of the plant symptomatic, and 5 = >50% of the plant symptomatic. Using this scale with assessments over time allowed us to monitor disease progress on individual plants. Sample isolations from dying plants were made twice to ensure that *S. rolfsii* was the pathogen causing disease. All data were subjected to statistical analysis using the general linear models procedure or regression procedures with PC-SAS. Differences reported as significant are at $P \leq 0.05$.

Results

Greenhouse Experiments. Three inoculation techniques consistently resulted in significantly high disease severity. Final disease severity was very similar for the agar disk and clothespin techniques in all greenhouse tests, GH1-4 (Fig. 1). The oat inoculum treatment caused slightly lower disease severities than the agar plug and clothespin techniques in GH1, 2, and 4 and was deleted from GH3 due to contamination with *Trichoderma* sp. Lower and more variable levels of disease resulted with the toothpick methods. Disease progress exhibited similar trends (Fig. 2). Rating curves for the agar disk, oat inoculum, and clothespin techniques var-

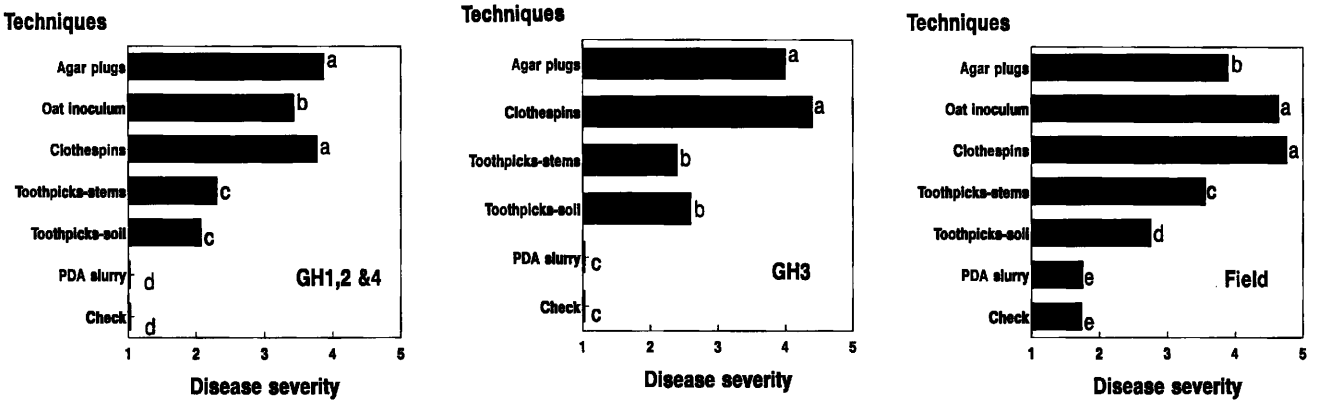

Fig. 1. A comparison of final disease ratings for stem rot inoculation techniques on peanut. Results for experiments GH1, 2, and 4 were similar and values shown represent the mean disease severity for each technique. Results of GH3 do not include the oat inoculation technique due to contamination in that experiment. A 1-5 disease rating scale was used in which 1 = healthy plant, 2 = lesions on stems only, 3 = up to 25% of the plant symptomatic (wilted, dead, or dying), 4 = 26-50% of the plant symptomatic, and 5 = >50% of the plant symptomatic.

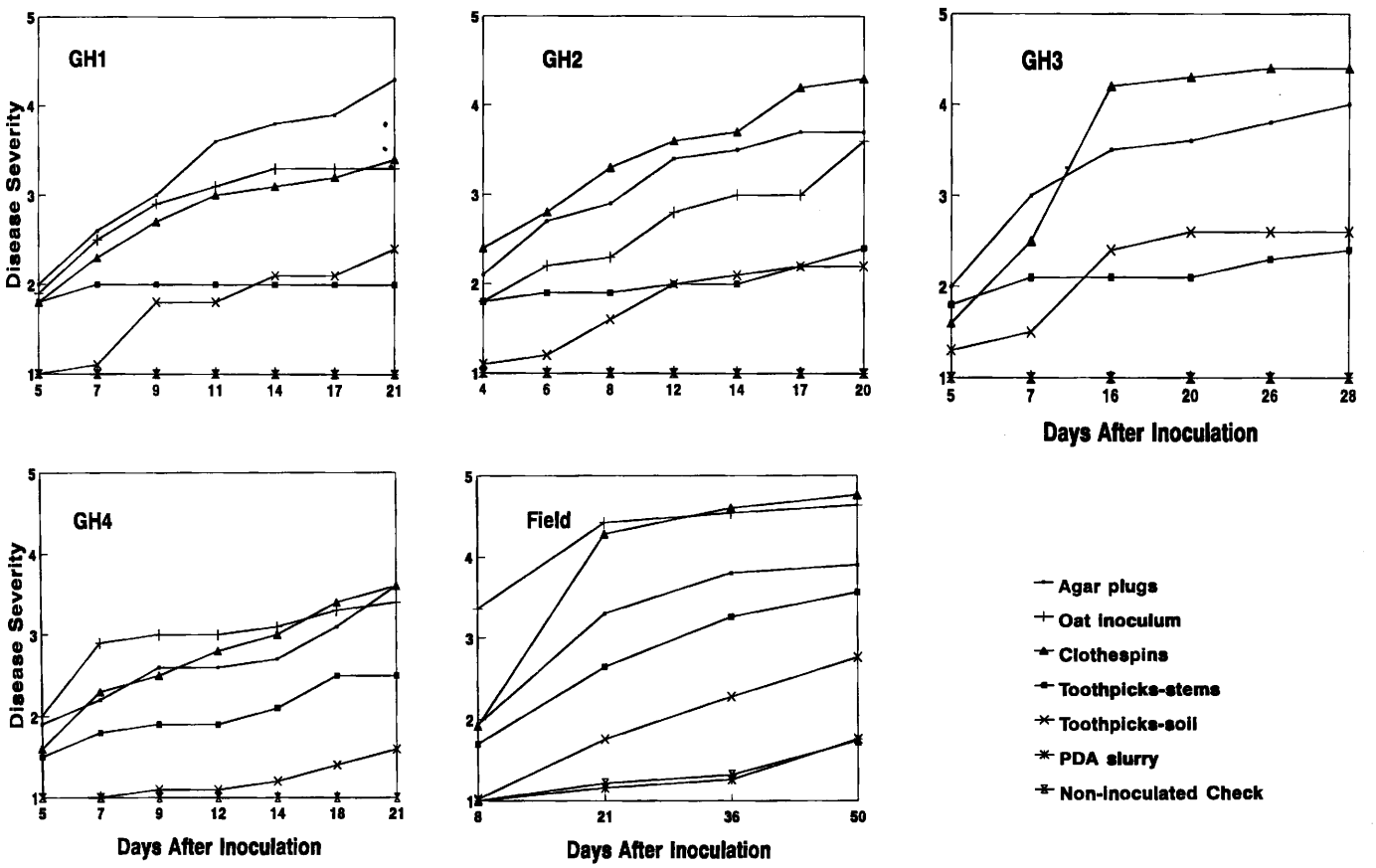

Fig. 2. A comparison of disease rating curves for stem rot inoculation techniques on peanut in four greenhouse experiments (GH1-GH4) and one field experiment. Oat inoculation in GH3 was deleted due to contamination. Where no curve is shown the disease rating was a 1.0 on the 1-5 disease rating scale in which 1 = healthy plant, 2 = lesions on stems only, 3 = up to 25% of the plant symptomatic (wilted, dead, or dying), 4 = 26-50% of the plant symptomatic, and 5 = >50% of the plant symptomatic.

ied from test to test but indicated a similar increase in disease over time. Areas under the disease rating curves were significantly greater for the agar disk, oat inoculum, and clothespin techniques in comparison to the other techniques (Table 1). No stem rot was noted on plants treated with the PDA slurry or the check plants treated with clean toothpicks in any of the greenhouse experiments.

Table 1. Mean area under disease rating curves for three greenhouse and one field experiment to compare inoculation techniques for inciting stem rot on peanut.

Technique	Greenhouse	Field
Agar plugs	36.8	84.4
Oat inoculum	32.9	115.7
Clothespins	36.1	110.9
Toothpicks: Stems	18.3	67.3
Toothpicks: Soil	10.4	37.3
PDA slurry	0	10.4
Check	0	11.7
LSD (0.05)	4.3	9.5

Field Experiment. The efficiency of the inoculation techniques in the greenhouse and field was similar for the oat inoculum and clothespin techniques (Fig. 1). The agar disk technique had significantly lower ratings in the field than either the oat inoculum or the clothespin techniques throughout the assessment period (Fig. 2). The toothpick-stem technique had higher ratings than the toothpick-soil technique, and both had significantly higher ratings than the untreated checks. The PDA-slurry technique, clean toothpicks, and the untreated checks all had a low disease rating (>1.0 but <2.0) in the field experiment. This was attributed to a low level of natural inoculum.

Correlation of Experiments. Regression of the final disease rating for all treatments in greenhouse experiments yielded significant coefficients of correlation that ranged from 0.841 to 0.978 (Table 2). The techniques of the field experiment were highly and significantly correlated with those in the greenhouse studies with correlation coefficients ranging from 0.798 to 0.971.

Table 2. Coefficients of correlation for final disease ratings of all techniques across all experiments.[a]

	GH1	GH2	GH3	GH4	Field
GH1		0.943	0.877	0.938	0.878
GH2			0.891	0.978	0.971
GH3				0.841	0.798
GH4					0.968

[a]All values are significant at $P \leq 0.05$.

Discussion

Results of these experiments clearly indicated that the agar plug, oat inoculum, and clothespin techniques were superior to the other methods. The mean final disease ratings for the agar disk and clothespin techniques were above 3.5 on the 1-5 scale in every test, and the oat inoculum technique was >3.5 in two tests and only slightly less in two others. Further confirmation of the consistency of these techniques was observed in disease progress and areas under the rating curves. Although there was some shifting of position for the three best techniques from one test to another, the overall results indicate that use of a given technique for stem rot inoculation depend more on the type of evaluation needed than on the anticipated reliability of the technique.

The agar disk technique may come closer to simulating natural infection since it involves actively growing mycelia, a germinated sclerotium, and a food base (the PDA disk). Using this technique, the plant is not wounded in any way and if conditions are favorable for infection it closely simulates the natural infection process. There is little opportunity for the plant to 'escape' the pathogen when using this technique and it minimizes interference by antagonistic soil microbes. Disadvantages are that agar disks are small, difficult to handle, and the procedure is time-consuming. Furthermore, agar disk inoculum may dry out if in direct sunlight and care must be taken in the field to ensure shading by the plant canopy. The agar disk technique is appropriate for single plant inoculatons, and we have used this technique with good success in 3 yr of evaluations for resistance to stem rot in peanut breeding lines (Shokes, unpubl. data).

The clothespin technique has advantages similar to the agar disk technique in that it provides actively growing mycelia and a food base. As with the agar disk technique there is little opportunity for disease escape and minimal probability of other microbial antagonism. If the clothespins are properly impregnated with PDB there may be greater inoculum potential (energy source) than with agar disks. Clothespins do little if any damage to the plant if applied carefully and they are easier to handle than agar disks or toothpicks. For these reasons we prefer this inoculation technique for single plant inoculations.

Oat inoculum has some advantages and has been used frequently by previous researchers. It is relatively easy to produce in volume and can be applied to large numbers of plants or large areas of a field. Difficulties with this method occur in the preparation process. It is difficult to sterilize oat seed thoroughly, and bacterial contamination may occur even after autoclaving twice. Another contamination problem can occur when hyperparasites, such as *Trichoderma* spp., are present. Precautions should be taken to ensure that all oat inoculum is placed under the canopy and out of direct sunlight. Oat inoculum allows deployment of mixtures of isolates to minimize effects of isolate variability. However, we found that there were no differences in results with one highly virulent isolate and a composite of virulent isolates (Shokes, unpubl. data). The oat inoculum technique allows changing the amounts of inoculum applied

by diluting with uninfested sterilized and dried oat seed or rolled oats. Such dilution provides an even greater food base for fungal growth and survival. The amounts of oat inoculum required to incite sufficient stem rot in the field can vary with conditions, and Shew *et al.* (16) reported that, when oat inoculum was used in field evaluations of resistance, peanut stem rot was affected by inoculum density independent of genotype. Further investigation of this is needed.

The toothpick-stem inoculation technique was less effective than anticipated. This technique has been retested with better results in another experiment (Shokes, unpubl. data). Like the agar disk and the clothespin techniques, the toothpick-stem technique is mainly for single plant inoculations. However, this technique has the undesirable effect of wounding plants and may bypass natural defense mechanisms in some peanut lines. The toothpick-stem technique did not cause high levels of disease in our studies and was not as effective as other methods.

The toothpick-soil technique was tried because it was perceived to be a rapid way to inoculate individual plants with minimum disturbance. The inconsistency of this method made it undesirable. A larger substrate with a greater volume of food source might be used to improve the method. Failure of the PDA-slurry technique to incite stem rot in our tests could be due to a rapid drying of the slurry.

The absence of disease in check plants (clean toothpicks and untreated) in the greenhouse studies indicates that controls were adequate. A low level of disease in the check plants in the field was expected since no effort was made to totally eliminate residual natural inoculum. The fact that disease in the checks was of minor consequence is an indication that this type of testing can be successful in the field when proper controls are used.

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